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Selected US specifications from IPC sub-class G01N

(54) Enzyme immunoassay

(57) An enzyme immunoassay is provided for the measurement of antigen, with improved sensitivity. Said immunoassay comprises the steps of:

(1) reacting [A] an immune-complex, obtained by reacting, at one time, (a) the antigenic substance, (b) an immobilized first antibody recognizing the antigenic substance and (c) a second antibody recognizing the antigenic substance originating from a different animal species, with [B] an enzyme-labelled third antibody recognizing immunoglobulin originating from the same animal species as said second antibody;

(2) separating a solid phase from the liquid phase; and

(3) measuring the amount of said enzyme present in the solid phase by means of the enzyme activity.

The antigen may be carcinoembryonic antigen, thyroid stimulation hormone, human chorionic gonadotropin beta sub unit or hepatitis B virus.

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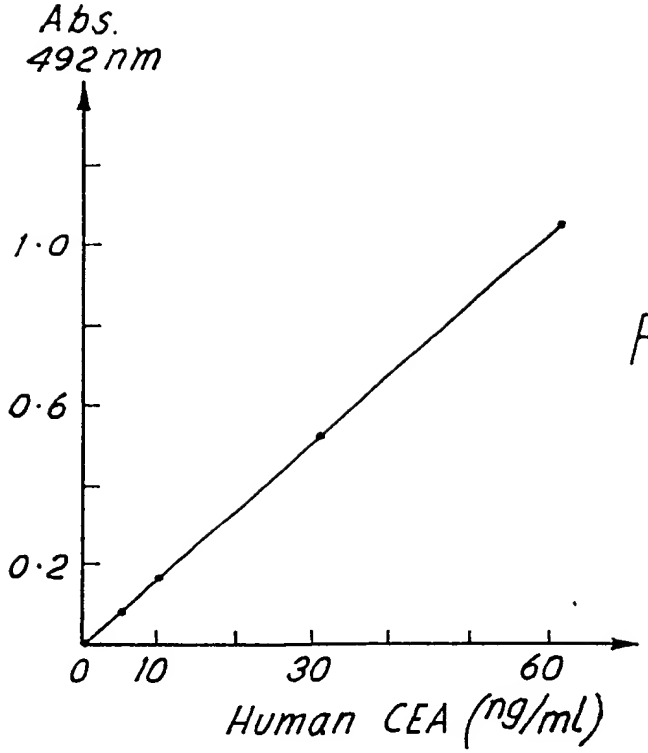

FIG.1

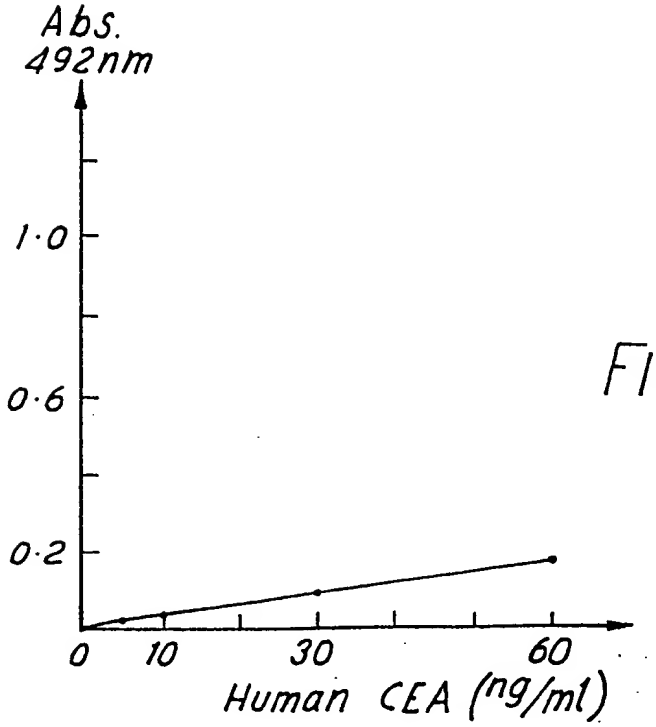

FIG.2

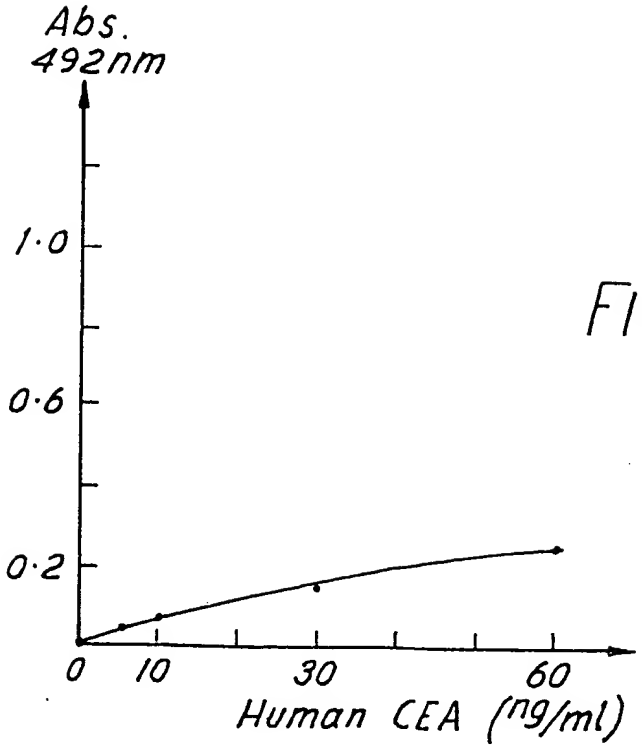
Abs.
492nm
1·0
0·6
0·2
0
10
30
60
Human CEA (ng/ml)

FIG. 3

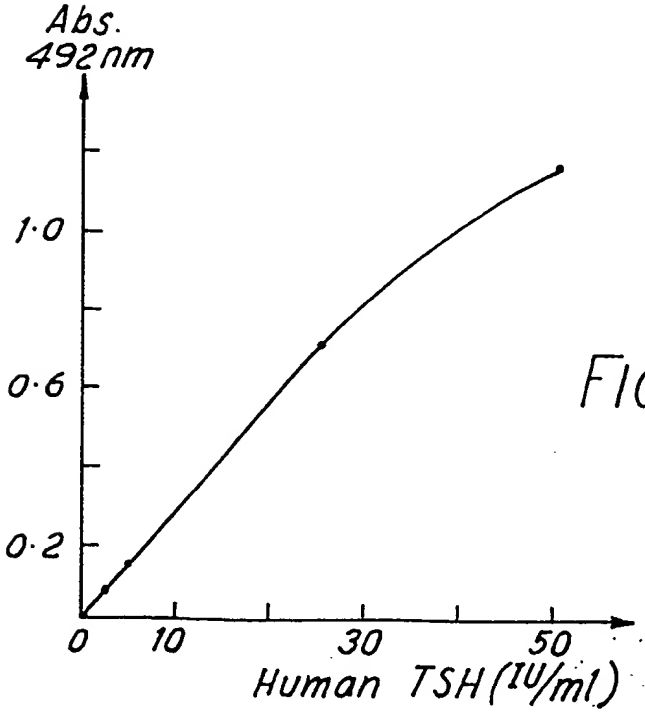
Abs.
492nm
1·0
0·6
0·2
0
10
30
50
Human TSH (IU/ml)

FIG. 4

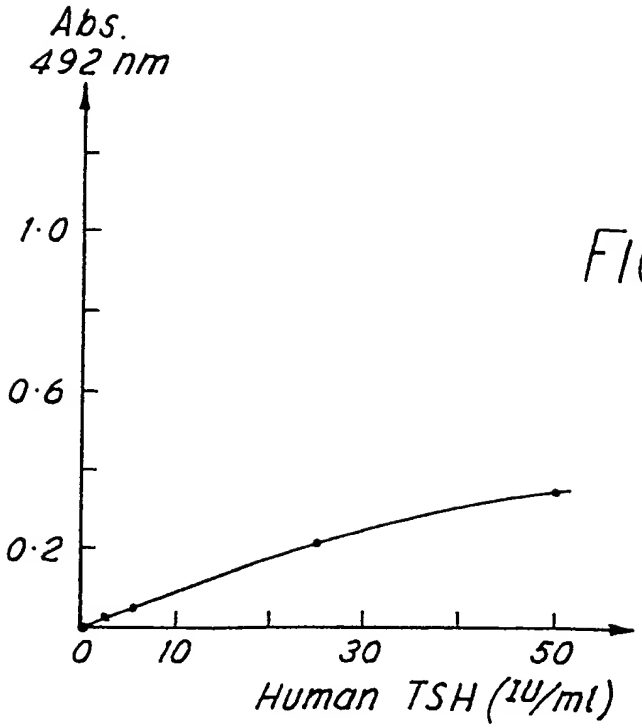
Abs.
492 nm
1·0
0·6
0·2
0
10
30
50
Human TSH (IU/ml)

FIG. 5

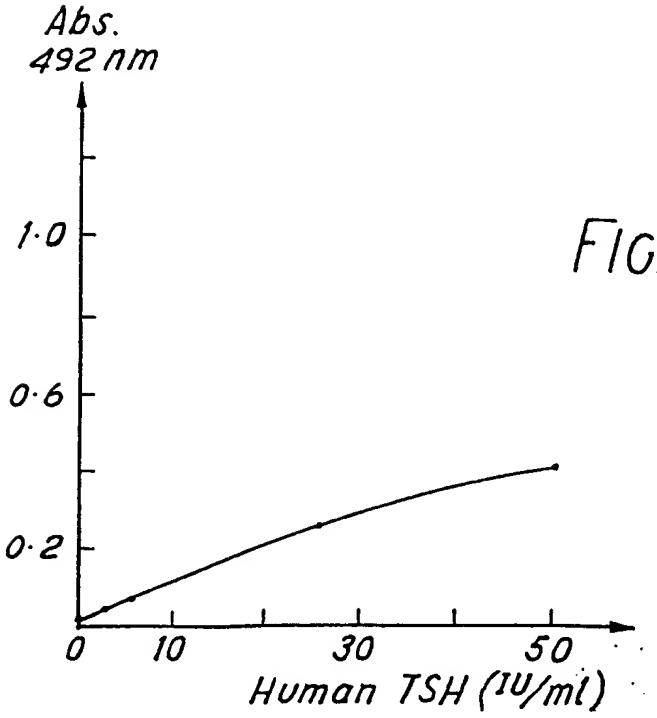
Abs.
492 nm
1·0
0·6
0·2
0
10
30
50
Human TSH (IU/ml)

FIG. 6

SPECIFICATION

Enzyme immunoassay

1. *Field of the invention:*
This invention relates to enzyme immunoassay (EIA).

2. *Description of the prior art:*
As an EIA technique, sandwich EIA has been known. This technique involves reacting an antigen bound to an immobilized first antibody with an enzyme-labelled second antibody, followed by measuring the amount of said enzyme (such as U.S.Pat.No. 4474878).
Such technique, however, has drawbacks that sufficient sensitivity is not obtained when titer of the second antibody is low.

Summary of the invention
It is an object of the present invention to provide an EIA having improved sensitivity.
It is another object of this invention to provide an EIA providing high sensitivity, even when titer of the second antibody is low.
It is still another object of the invention to provide an EIA capable of using an unpurified antibody as the second antibody with sufficient high sensitivity.
It is yet another object of the invention to provide an EIA, which can attain measurement of high specificity to antigen.
Briefly, these and other objects of the present invention as hereinafter will become more readily apparent have been attained broadly by a method of enzyme immunoassay for quantitating an antigenic substance, which comprises the steps of:
(1) reacting
[A] an immune-complex, obtained by reacting, at one time, (a) the antigenic substance, (b) an immobilized first antibody recognizing the antigenic substance and (c) a second antibody recognizing the antigenic substance originating from a different animal species, with
[B] an enzyme-labelled third antibody recognizing immunoglobulin originating from the same animal species as said second antibody;
(2) separating a solid phase from the liquid phase; and
(3) measuring the amount of said enzyme present in the solid phase by means of the enzyme activity.

Brief description of the drawings
Figures 1, 2, 3, 4, 5 and *6* are EIA standard curves obtained in Example 1, Comparative Example 1, Comparative Example 2, Example 2, Comparative Example 3 and Comparative Example 4, respectively.

Detailed description of the preferred embodiments
Illustrative examples of suitable antigenic substances employed in the present invention, are
1) hormones, such as insulin, human chorionic gonadotropin beta-subunit (HCG-beta), growth hormone, thyroid stimulating hormone (TSH), thyroxine, triiodothyronine, and the like;
2) serum proteins, such as IgG, IgA, IgM, IgE, alpha-fetoprotein, $beta_2$-microglobulin, TBG, and the like;
3) tumor associated antigens, such as carcinoembryonic antigen (CEA), ferritin, POA, CA-19-9, CA125, and the like; and
4) pathogens (pathogenic bacteria, viruses, parasites or protozoas, causing various disease), such as streptococcus, hepatitis viruses (such as hepatitis B virus), rubella virus, herpes virus, toxoplasma gondii, malarial parasite and the like.
Among these antigens, preferred are HCG-beta, TSH, CEA and hepatitis B virus, with respect to high sensitivity.
Suitable first antibodies, usable in this invention, include polyclonal antibodies and monoclonal antibodies. Polyclonal antibodies can be obtained by immunizing a mammal (such as rabbit, goat, sheep, guinea pig and the like) with the antigen. Monoclonal antibodies useful in the present invention can be obtained by known process, as described in Nature *256*, 495–497, for example. Basically, it involves injecting a mouse or other suitable animal with an immunogen, fusing antibody-producing cells taken from the animal with myeloma cells (originated from a mouse of other suitable animal), and culturing or asciting the resulting hybridoma or hybrid cell. These antibodies may be purified by known methods, such as ammonium-sulfate precipitation, DEAE-cellulose chromatography, affinity chromatography and the like.
Said first antibody, in the invention, can be immobilized on an inorganic or organic carrier (insoluble solid). Suitable carriers include inorganic carriers, for example, siliceous materials [such as glass (porous glass, frosted glass and so on), silica (silica gel, colloidal silica), bentonite, wollastonite, cordierite and the like], and nonsiliceous metal oxides [such as alumina, spinel, apatite, hydroxy apatite, titania, zirconia, and magnetic substances (such as iron oxides, ferrite, nickel oxides, cobalt oxides and the like)]; and organic carriers, for instance, plastics [such as polystyrene, and derivatives thereof, such as poly(aminostyrene); acrylic polymers, such as polyacrylonitrile; polymethacrylates, such as polymethylmethacrylate; polyolefines, such as

polyethylene, polypropylene, polybutene and polybutadiene; halogen-containing polymers, such as poly-(vinyl chloride) and poly(vinylidene chloride); polyesters, such as poly(ethylene terephthalate); polyamides, such as nylon 6 and nylon 6,6; and so on]; and natural polymers, such as polysaccharides, cellulose, dextran, agarose, paper (such as filter paper), polypeptide, collagen and the like.

These carriers may be particulate in nature, varying from a finely divided powder to a coarse granular material (e.g. about 20–about 100 mesh or more, U.S. Standard Sieve), or may be a shaped article, such as sheet or pellet or three-dimensional articles, such as beads, test tubes, trays, discs and so on. Among these, preferred are glass (particularly glass beads and glass test tubes) and plastics (plastic tubes and plastic trays). These carriers may be porous, or surface-modified by known methods, such as etching or frosting, chemical treatment, chemical coating and the like.

The antibody can be immobilized by any known means, which can vary from simple adsorption to chemical coupling. Chemical coupling typically involves treating the carrier with one or more chemical compounds (silanes, polyisocyanates and the like), followed by contacting the treated carrier with an aqueous solution of the antibody. Adsorption usually involves contacting an aqueous solution of the antibody to be immobilized with the carrier for a time sufficient to permit the desired or maximum degree of immobilization. Examples of procedures suitable for immobilization of the antibody are those by physical adsorption or chemical coupling to glass with a silane coupling agent with or without a crosslinking agent, as described in U.S.Pat.No.4,280,992; and those by physical adsorption to plastics, as written in E.Engvall, J.Johnson, P.Parlman: Biochim. Biophys, Acta, *251* (1971) 427–434.

Examples of suitable second antibodies include:

1) polyclonal antibodies, recognizing the antigen and originating from an animal species different from that of the first antibody [for example, antibodies originating from goat, sheep, guinea pig and the like (in case of the first antibody originating from rabbit), and antibodies from rabbit, goat, guinea pig and the like (in case of the first antibody from sheep)]; and

2) monoclonal antibodies, produced by culturing or asciting the resulting hybridoma or hybrid cell, obtained by fusing antibody-producing cells against the antigen (originating from a mouse, for example) with myeloma cells (originating from a mouse) in case where the first antibody is polyclonal antibody recognizing the antigen and originating from an animal (other than a mouse).

Suitable forms of second antibodies include, for example, anti-sera, hybridoma culture fluid and ascites fluid, as well as immunoglobulin obtainable by purifying them.

Concentration of immunoglobulin, which may vary widely according to concentration of the antigen in the sample to be tested and concentration of the enzyme-labelled third antibody, is generally from about 0.1 to about 1000 microgram/ml, preferably from about 1 to about 200 microgram/ml.

Said immuno-complex can be obtained, according to the present invention, by reacting the immobilized first antibody, the antigenic substance and the third antibody at the same time. For instance, a first antibody bound on an insoluble carrier, an antigen-containing sample to be tested, and a second antibody-containing buffer are incubated coincidently to obtain an immuno-complex. Incubation can be carried out under usual conditions, for example, 5–50°C, preferably 34–40°C, for 5 minutes–2 days, preferably 5–30 minutes.

After the incubation, unreacted materials may be removed from the resulting immuno-complex, in the usual way. For example 1–5 ml of a washing liquid (such as distilled water, physiologic saline, a phosphate buffer or the like) is added to the incubated mixture, followed by removing the liquid under suction using an aspirator. The procedure is repeated several times (for example, twice–five times) to obtain an immuno-complex separated from unreacted materials.

Examples of suitable third antibodies are antibodies recognizing immunoglobulin originating from the same animal species as the second antibody: for example, anti-rabbit immunoglobulin antibody (guinea pig) in case where the second antibody is antibody originating from rabbit; anti-goat immunoglobulin antibody (mouse) in case of the second antibody from goat; and anti-mouse immunoglobulin antibody (rabbit) in case of the second antibody from mouse.

As to combinations of the first, second and third antibodies, it is preferred, in view of lower non-specific adsorption, to use the third antibody originating from the same animal species as the first antibody. Illustrative examples of combinations of the first, second and third antibodies are: (1) the first antibody from rabbit, the second monoclonal antibody from mouse and third antibody from rabbit; (2) the first antibody from guinea pig, the second antibody from sheep and third antibody from guinea pig; (3) the first antibody from sheep, the second antibody from rabbit and third antibody from sheep; and (4) the first antibody from goat, the second monoclonal antibody from mouse and third antibody from goat. Among these, (1) and (4) are preferred, with respect to higher specificity against the antigen.

Suitable enzymes, for labelling the third antibody, are inclusive of peroxidase, alkaline phosphatase, beta-galactosidase, and the like. Among these, more preferred is peroxidase, which can provide easy labelling of antibody and high sensitivity.

Labelling of the third antibody may be performed by any known methods, for example, those described in S.Yoshitake, M.Imagawa, E.Ishikawa, et al, J. Biochem. *92* (1982) 1413–1424.

Reaction of the enzyme-labelled antibody with the immuno-complex obtained by concurrent reaction according to this invention can be performed in the usual ways. For instance, the immuno-complex is transferred into 100–1000 micro-litres of a solution of the enzyme-labelled antibody, followed by incubating the mixture. The incubation may be carried out under usual conditions, for example, 5–50°C, preferably

34–40°C for 5 minutes–2 days, preferably 5–30 minutes.

The reaction product of the immuno-complex with the enzyme-labelled antibody may be washed with a washing liquid (such as distilled water, physiologic saline, a phosphate buffer or the like). After washing, the product is transferred into 100–1000 micro-litres of a substrate [such as 5-amino-salicylic acid, o-phenylene diamine, 2,2'-azinodi(3-ethylbenzthiazoline)-6'-sulfonic acid (ABTS) or the like, preferably o-phenylene diamine], followed by incubation. The incubation may be carried out under usual conditions, for example, 5–50°C, preferably 30–40°C, for 5 minutes–1 hour, preferably 5–30 minutes. After incubation, 1–10 ml of a reaction terminator (such as sulfuric acid, hydrochloric acid or the like) is added to the incubated mixture. The product thus obtained is used for measurement of enzyme activity.

Having generally described the invention, a more complete understanding can be obtained by reference to certain specific examples, which are included for purposes of illustration only and are not intended to be limiting unless otherwise specified.

EXAMPLE 1 [Measurement of CEA]

a) *Preparation of Human CEA standard solution*

Concentration of a high concentration CEA solution, obtained from metastatic colonic tumours by perchloric acid extraction, was detected by using CEA International Reference Standard (63/701) of WHO, with use of a CEA-measuring kit [CEA-EIA, produced by DAINABOT], followed by diluting the solution with 0.02 M phosphate buffer to obtain standard solutions of 5, 10, 30 and 60 ng/ml, respectively.

b) *Preparation of Anti-CEA monoclonal antibody*

A mouse (Balb/c) was immunized by injecting with a high concentration human CEA solution. After 6 weeks, a cell suspension was produced from the spleen; and then about 1×10^8 cells of the spleen and about 2×10^7 mouse myeloma cells were fused with PEG treatment. The resulting fused cells were cultured in HAT medium, followed by screening to select antibody-producing cells (hybridoma). Afterwards, this hybridoma was grown as monoclone by cloning, followed by asciting the monoclone with a mouse. The resulting ascites fluid was purified to obtain an anti-CEA monoclonal immunoglobulin.

c) *Preparation of Peroxidase-labelled anti-mouse immunoglobulin antibody*

An anti-mouse immunoglobulin antibody (produced by Dako) was combined with peroxidase according to the method described in J. Biochem. *92* (1982), 1413–1424, followed by diluting the resultant to 1/10–1/5000 concentration with a buffer containing 1 % bovine serum albumin.

d) *Preparation of Glass beads coated with anti-CEA polyclonal antibodies*

Anti-CEA polyclonal rabbit antibody (produced by Dako) were coated onto the surface of glass beads, according to the method of U.S.Pat.No.3652761.

e) *Quantitation of Human CEA*

One glass bead coated with rabit anti-CEA polyclonal antibodies and 50 micro-litres of 60 ng/ml human CEA standard solution were incubated for 15 minutes at 37°C in 300 micro-litres of 0.02 M phosphate buffer containing 10–100 micro-grams/ml anti-CEA monoclonal antibody, within a test tube, followed by washing the bead with distiled water. Then, the bead was removed into 300 micro-litres of peroxidase-labelled anti-mouse immunoglobulin antibody solution, followed by incubating for 15 minutes at 37°C. After washing the bead again with distiled water, the bead was incubated for 15 minutes at 37°C in 400 micro-litres of a substrate solution (o-phenylene diamine solution containing hydrogen peroxide). Thereafter, the reaction was terminated by adding 3 ml of 1.5 N sulfuric acid. Absorbance at 492 nm of the resulting solution was measured to quantitate the enzyme-activity of the enzyme bound to the bead.

Enzyme-activities in case of human CEA standard solutions of 30, 10, 5 and 0 ng/ml, respectively, were measured in the same manner, to make a standard curve for assaying human CEA, as shown in Figure 1.

The results of assaying human CEA were as shown in Table 1.

COMPARATIVE EXAMPLE 1 [Measurement of CEA by Sandwich Method]

a) *Preparation of Human CEA standard solution*

Example 1, a) was repeated.

b) *Preparation of anti-CEA monoclonal immunoglobulin*

Example 1, b) was repeated.

c) *Preparation of Peroxidase-labelled anti-CEA monoclonal immunoglobulin*

The above anti-CEA monoclonal immunoglobulin was combined with peroxidase in the same way as Example 1,c), followed by diluting the resultant to 1/10–1/5000 conc. with a buffer containing 1 % bovine serum albumin.

d) *Preparation of Glass beads coated with anti-CEA polyclonal antibodies*

Example 1, d) was repeated.

e) *Quantitation of Human CEA*

One glass bead coated with anti-CEA polyclonal rabbit antibodies and 50 micro-litters of 60 ng/ml human CEA standard solution were incubated for 15 minutes at 37°C in 200 micro-litres of 0.02 M phosphate buffer, within a test tube, followed by washing the bead with distilled water. Then, the bead was removed into 300 micro-litres of peroxidase-labelled anti-CEA monoclonal immunoglobulin solution, followed by incubating for 15 minutes at 37 C°. After washing the bead again with distilled water, the bead was incubated for 15 minutes at 37°C in 400 micro-litres of a substrate solution (o-phenylene diamine solution containing hydrogen peroxide). Thereafter, the reaction was terminated by adding 3 ml of 1.5 N sulfuric acid. Absorbance at 492 nm of the resulting solution was measured to quantitate the enzyme-activity of the enzyme bound to the bead.

Enzyme-activities in case of human CEA standard solutions of 30, 10, 5 and 0 ng/ml, respectively, were measured in the same manner, to make a standard curve for assaying human CEA, as shown in Figure 2.

The results of assaying human CEA were as shown in Table 1.

COMPARATIVE EXAMPLE 2 [Measurement of CEA by successive reaction method]

a) *Preparation of Human CEA standard solution*

Example 1, a) was repeated.

b) *Preparation of Anti-CEA monoclonal immunoglobulin*

Example 1, b) was repeated.

c) *Preparation of Peroxidase-labelled anti-CEA monoclonal immunoglobulin*

Example 1, c) was repeated.

d) *Preparation of Glass beads coated with anti-CEA polyclonal antibodies*

Example 1, d) was repeated.

e) *Quantitation of Human CEA*

One glass bead coated with rabbit anti-CEA polyclonal antibodies and 50 micro-litres of 60 ng/ml human CEA standard solution were incubated for 15 minutes at 37°C in 300 micro-litres of 0.02 M phosphate buffer, within a test tube, followed by washing the bead with distiled water. Then, the bead was removed into 300 micro-litres of 0.02 M phosphate buffer containing 10–100 micro-grams/ml of anti-CEA monoclonal antibody, followed by incubating for 15 minutes at 37°C. After washing the bead with distilled water, the bead was removed into 300 micro-litres of peroxidase-labelled anti-CEA monoclonal immunoglobulin solution, followed by incubating for 15 minutes at 37°C. After washing the bead again with distilled water, the bead was incubated in 400 micro-litres of a substrate solution (o-phenylene diamine solution containing hydrogen peroxide). Thereafter, the reaction was terminated by adding 3 ml of 1.5 N sulfuric acid. Absorbance at 492 nm of the resulting solution was measured to quantitate the enzyme-activity of the enzyme bound to the bead.

Enzyme-activities in case of human CEA standard solutions of 30, 10, 5 and 0 ng/ml, respectively, were measured in the same manner, to make a standard curve for assaying human CEA, as shown in Figure 3.

The results of assaying human CEA were as shown in Table 1.

TABLE 1

	Sensitivity ng/ml	*Assay range ng/ml*	*Precision ng/ml (coefficient of variation, %)*	*ng/ml (coefficient of variation, %)*
Example 1	1.0	0–60	5.0±0.4 (8.0%)	38.2±2.2 (5.8%)
Comparative Example 1	4.5	0–60	5.3±0.9 (17.0%)	37.5±5.3 (14.1%)
Comparative Example 2	3.9	0–60	6.6±0.9 (13.6%)	40.2±5.8 (14.4%)

EXAMPLE 2 [Measurement of TSH]

a) *Preparation of Human TSH standard solution*

Human TSH (50 micro-grams/vial), obtained from UCB Bioproducts, was diluted with 0.02 M phosphate buffer to obtain standard solutions of 50, 25, 5, 2.5 and 0 IU/ml, respectively.

b) *Preparation of Anti-TSH monoclonal antibody*

Example 1, b) was repeated as above except human TSH was used instead of human CEA to obtain an anti-TSH monoclonal antibody.

c) *Preparation of Glass beads coated with anti-TSH polyclonal antibodies*

Example 1, d) was repeated except using Anti-TSH polyclonal rabbit antibody (produced by Dako) instead of

anti-CEA polyclonal rabbit antibody.

d) *Quantitation of Human TSH*

One glass bead coated with anti-TSH polyclonal rabbit antibodies and 100 micro-litres of 50 IU/ml human TSH standard solution were incubated for 30–60 minutes at 37°C in 200 micro-litres of 0.02 M phosphate buffer containing 10–100 micro-grams/ml anti-TSH monoclonal antibody, within a test tube, followed by washing the bead with distiled water. Then, the bead was removed into 300 micro-litres of peroxidase-labelled anti-mouse immunoglobulin antibody solution prepared in Example 1, c), followed by incubating for 30–60 minutes at 37°C. After washing the bead again with distiled water, the bead was incubated for 30 minutes at 10–30°C in 400 micro-litres of a substrate solution (o-phenylene diamine solution containing hydrogen peroxide). Thereafter, the reaction was terminated by adding 3 ml of 1.5 N sulfuric acid. Absorbance at 492 nm of the resulting solution was measured to quantitate the enzyme-activity of the enzyme bound to the bead.

Enzyme-activities in case of human TSH standard solutions of 25, 5, 2.5 and 0 IU/ml, respectively, were measured in the same manner, to make a standard curve for assaying human TSH, as shown in Figure 4.

The results of assaying human TSH were as shown in Table 2.

COMPARATIVE EXAMPLE 3 [Measurement of TSH by Sandwich Method]

a) *Preparation of Human TSH standard solution*

Example 2, a) was repeated.

b) *Preparation of Anti-TSH monoclonal immunoglobulin*

Example 2, b) was repeated.

c) *Preparation of Peroxidase-labelled anti-TSH monoclonal immunoglobulin*

The above anti-TSH monoclonal immunoglobulin was combined with peroxidase in the same way as Example 1, c), followed by diluting the resultant to 1/10–1/5000 conc. with a buffer containing 1 % bovine serum albumin.

d) *Preparation of Glass beads coated with anti-TSH polyclonal antibodies*

Example 2, c) was repeated.

e) *Quantitation of Human TSH*

One glass bead coated with anti-TSH polyclonal antibodies and 100 micro-litres of 50 IU/ml human TSH standard solution were incubated for 60 minutes at 37°C in 200 micro-litres of 0.02 M phosphate buffer containing 10–100 micro-grams of anti-TSH monoclonal antibody, within a test tube, followed by washing the bead with distiled water. Then, the bead was removed into 300 micro-litres of peroxidase-labelled anti-TSH monoclonal immunoglobulin solution, followed by incubating for 60 minutes at 37°C. After washing the bead again with distiled water, the bead was incubated for 30 minutes at 37°C in 400 micro-litres of a substrate solution (o-phenylene diamine solution containing hydrogen peroxide). Thereafter, the reaction was terminated by adding 3 ml of 1.5 N sulfuric acid. Absorbance at 492 nm of the resulting solution was measured to quantitate the enzyme-activity of the enzyme bound to the bead.

Enzyme-activities in case of human TSH standard solutions of 25, 5, 2.5 and 0 IU/ml, respectively, were measured in the same manner, to make a standard curve for assaying human TSH, as shown in Figure 5.

The results of assaying human TSH were as shown in Table 2.

COMPARATIVE EXAMPLE 4 [Measurement of TSH by successive reaction method]

a) *Preparation of Human TSH standard solution*

Example 2, a) was repeated.

b) *Preparation of Anti-TSH monoclonal immunoglobulin*

Example 2, b) was repeated.

c) *Preparation of Glass beads coated with anti-TSH polyclonal antibodies*

Example 2, c) was repeated.

d) *Quantitation of Human CEA*

One glass bead coated with rabbit anti-TSH polyclonal antibodies and 100 micro-litres of 50 IU/ml human TSH standard solution were incubated for 30 minutes at 37°C in 200 micro-litres of 0.02 M phosphate buffer, within a test tube, followed by washing the bead with distilled water. Then, the bead was removed into 300 micro-litres of 0.02 M phosphate buffer containing 10–100 micro-grams/ml of anti-TSH monoclonal antibody, followed by incubating for 30 minutes at 37°C. After washing the bead with distiled water, the bead was removed into 300 micro-litres of peroxidase-labelled anti-mouse immunoglobulin antibody solution, followed by incubating for 30 minutes at 37°C. After washing the bead again with distilled water, the bead was incubated in 400 micro-litres of a substrate solution (o-phenylene diamine solution containing hydrogen peroxide). Thereafter, the reaction was terminated by adding 3 ml of 1.5 N sulfuric acid. Absorbance at 492 nm

of the resulting solution was measured to quantitate the enzyme-activity of the enzyme bound to the bead.

Enzyme-activities in case of human TSH standard solutions of 25, 5, 2.5 and 0 IU/ml, respectively, were measured in the same manner, to make a standard curve for assaying human TSH, as shown in Figure 6.

The results of assaying human TSH were as shown in Table 2.

TABLE 2

	Sensitivity IU/ml	*Assay range IU/ml*	*Precision IU/ml (coefficient of variation, %)*	*IU/ml (coefficient of variation, %)*
Example 2	2.0	0–50	2.8±0.2 (7.1%)	26.0±1.4 (5.4%)
Comparative Example 3	6.5	0–50	2.6±0.7 (26.9%)	25.5±4.3 (16.9%)
Comparative Example 4	5.7	0–50	3.0±0.6 (20.0%)	28.2±4.7 (16.6%)

EIA according to the present invention provides sufficiently high sensitivity, even if titer of the second antibody is low; while EIA by sandwich method and EIA by three antibody successive reaction method result in poor sensitivity, especially when titer of the second antibody is low. This invention can provide high sensitivity, even when the second antibody is unpurified one, such as anti-sera, culture fluid or ascites fluid. In addition, high sensitivity can be obtained according to the invention, even when monoclonal antibody is used as the second antibody; whereas monoclonal antibodies generally have high specificity but poor affinity and therefore poor sensitivity. Accordingly, measurement of high specificity with high sensitivity can be attained according to the invention, by using monoclonal antibody as the second antibody. Thus, the present invention are particularly useful as diagnostic reagents for CEA, TSH, HCG-beta and HBs antigens, in which high sensitivity is required.

CLAIMS

1. An method of enzyme immunoassay for quantitating an antigenic substance, which comprises the steps of:

(1) reacting

[A] an immune-complex, obtained by reacting, at one time, (a) the antigenic substance, (b) an immobilized first antibody recognizing the antigenic substance and (c) a second antibody recognizing the antigenic substance originating from a different animal species, with

[B] an enzyme-labelled third antibody recognizing immunoglobulin originating from the same animal species as said second antibody;

(2) separating a solid phase from the liquid phase; and

(3) measuring the amount of said enzyme present in the solid phase by means of the enzyme activity.

2. A method as claimed in Claim 1, wherein the second antibody is a monoclonal antibody.

3. A method as claimed in Claim 1 or 2, wherein the enzyme is peroxidase.

4. A method as claimed in Claim 1, 2 or 3, wherein the antigen is carcinoembryonic antigen, thyroid stimulatinm hormone, human chorionic gonadotropin beta-subunit or hepatitis B virus.

5. A method as claimed in any one of Claims 1–4, wherein the third antibody originate from the same animal species as the first antibody.

6. A method as claimed in any one of Claims 1–5, wherein the first antibody is immobilized on an inorganic carrier.

7. A method as claimed in Claim 6, wherein the carrier is siliceous.

8. A method as claimed in Claim 7, wherein the carrier is glass.

9. A method as claimed in Claim 6, 7 or 8, wherein the carrier is particulate and relatively finely divided.

10. A method as claimed in any one of Claims 1–5, wherein the first antibody is immobilized on an organic carrier.

11. A method as claimed in Claim 10, wherein the carrier is plastic.

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